



Evaluation of a PK/PBAN analog with an (*E*)-alkene, *trans*-Pro isostere identifies the Pro orientation for activity in four diverse PK/PBAN bioassays

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ABSTRACT

The pyrokinin/pheromone biosynthesis activating neuropeptide (PK/PBAN) family plays a multifunctional role in an array of important physiological processes in a variety of insects. An active core analog containing an (*E*)-alkene, *trans*-Pro isosteric component was evaluated in four disparate PK/PBAN bioassays in four different insect species. These bioassays include pheromone biosynthesis in the moth *Heliothis peltigera*, melanization in the larval *Spodoptera littoralis*, pupariation acceleration in the larval fly *Neobellieria bullata*, and hindgut contraction in the cockroach *Leucophaea maderae*. The conformationally constrained analog demonstrated activity equivalent to parent PK/PBAN peptides of equal length in all four PK/PBAN bioassays, and matched and/or approached the activity of peptides of natural length in three of them. In the melanization bioassay, the constrained analog exceeded the efficacy (maximal response) of the natural PBAN1-33 by a factor of 2 (at 1 nmol). The results provide strong evidence for the orientation of Pro and the core conformation adopted by PK/PBAN neuropeptides during interaction with receptors associated with a range of disparate PK/PBAN bioassays. The work further identifies a scaffold with which to design mimetic PK/PBAN analogs as potential leads in the development of environmentally favorable pest management agents capable of disrupting PK/PBAN-regulated systems.

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1. Introduction

The pyrokinin/pheromone biosynthesis activating neuropeptide (PK/PBAN) family of peptides plays a multifunctional role in the physiology of insects. In 1986 the first member of the family, leucopyrokinin (LPK), was isolated from the cockroach *Leucophaea maderae* [12] with over 30 members of this peptide class identified thereafter. All family members share the common C-terminal pentapeptide FXPRL-amide (X=S, T, G or V) and include subfamilies such as PKs, myotropins (MTs), PBAN, diapause hormone (DH), melanization and reddish coloration hormone (MRCH), pheromonotropin (PT), as well as pheromonotropic β and γ peptides derived from the cDNA of moths [2,26,27]. The PK/PBAN family has been shown to stimulate sex pheromone biosynthesis in

moths [2,26,27,29], and mediate critical functions associated with feeding (gut contractions) [19,30], development (egg diapause, pupal diapause and pupariation) [13,20,25,35,36], defense, molting and development (melanin biosynthesis) [4,15] in a variety of insects. The peptides do not exhibit species specificity and experiments have shown that all of the functions listed above can be stimulated by more than one peptide [1,6,10,26,27], and that the C-terminal pentapeptide common to the PK/PBAN neuropeptide class retains activity in each of the disparate functions. The functional diversity of the PK/PBAN family raises many questions regarding the mechanisms by which these neuropeptides operate and structural features PK/PBAN ligands hold in common during interaction with the receptors associated with the wide variety of physiological processes enumerated above.

In previous work, a highly rigid cyclic PK/PBAN analog *cyclo*[Asn¹]LPK (*cyclo*[NTSFTPTL]), featuring a *trans*-Pro, type I β -turn, was determined to retain significant bioactivity in several PK/PBAN bioassays, including hindgut contractile (cockroach *L. maderae*) [23], oviduct contractile (cockroach *L. maderae*) [22],

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pheromonotropic (silk worm *Bombyx mori*) [21], egg diapause induction (silk worm *B. mori*) [22], and pupariation (flesh fly *Neobellieria bullata*) [36] assay systems. These results are consistent with the suggestion that a *trans*-oriented Pro and the type I β -turn structure holds broad significance for many physiological functions elicited by the PK/PBAN family of peptides.

In this manuscript, we seek definitive evidence of the importance of a *trans*-oriented Pro for the whole spectrum of PK/PBAN bioactivities by incorporating an (*E*)-alkene, *trans*-Pro isostere (Ser- $\Psi[(E)\text{-CH=C}]$ Pro), that locks in the *trans*-Pro orientation, into a PK/PBAN C-terminal hexapeptide analog and evaluating it in four different PK/PBAN bioassays; pheromone biosynthesis in the moth *H. peltigera*, melanization in the Egyptian cotton leaf worm *S. littoralis*, pupariation in the fleshfly, *N. bullata*, and hindgut contraction in the cockroach *L. maderae*. The study further evaluates whether the (*E*)-alkene, *trans*-Pro isostere could represent a scaffold with which to design pseudopeptide and/or nonpeptide PK/PBAN mimetic analogs that may disrupt a range of critical PK/PBAN processes in pest insects.

2. Materials and methods

The PK/PBAN truncated analog 1559 was synthesized as described previously [18].

2.1. Synthesis of Fmoc-Ser(OTBDMS) $\Psi[(E)\text{-CH=C}]$ Pro-OH

The protected motif Fmoc-Ser- $\Psi[(E)\text{-CH=C}]$ Pro-OH (TBDMS = *tert*-butyldimethylsilyl; Ψ indicates that the following peptide linkage features a peptidomimetic modification: the nature of this modification is provided in the bracket; *E* = *trans*) was synthesized as previously described by Wang et al. [33,34]. Fmoc-Ser- $\Psi[(E)\text{-CH=C}]$ Pro-OH (465 mg, 1.12 mmol) and imidazole (381 mg, 5.60 mmol) were dissolved in DMF (4.0 ml), and TBDMSCl (422 mg, 2.80 mmol) was added. The mixture was stirred for 16 h, and then NH_4Cl (20 ml) was added. The mixture was stirred for an additional 50 min, and then diluted with EtOAc (30 ml), washed with NH_4Cl (2×10 ml), dried with MgSO_4 , and concentrated. Chromatography on silica gel with 0.1% acetic acid/30% EtOAc/hexanes gave 450 mg (76%) of Fmoc-Ser- $\Psi[(E)\text{-CH=C}]$ Pro-OH as a colorless foam. m.p. 62–63 °C. ^1H NMR (DMSO- d_6) δ 7.88 (d, *J* = 7.4, 2H), 7.68 (d, *J* = 7.4, 2H), 7.41 (t, *J* = 7.5, 2H), 7.31 (t, *J* = 7.2, 2H), 7.28 (d, *J* = 8.5, 1H), 5.37 (d, *J* = 7.6, 1H), 4.27 (m, 2H), 4.16 (m, 2H), 3.50 (dd, *J* = 10.1, 6.7, 1H), 3.40 (dd, *J* = 9.9, 6.7, 1H), 3.17 (t, *J* = 7.1, 1H), 2.35 (m, 1H), 2.26 (m, 1H), 1.80 (m, 3H), 1.53 (m, 1H), 0.82 (s, 9H), -0.01 (d, *J* = 2.8, 6H). ^{13}C NMR (DMSO- d_6) δ 175.2, 156.2, 144.6, 144.5, 141.3, 128.1, 127.6, 125.8, 121.3, 120.7, 65.9, 65.3, 53.1, 49.6, 47.3, 30.1, 29.7, 26.3, 25.0, 18.5, -4.8 , -4.9 . Anal. Calcd. for $\text{C}_{30}\text{H}_{39}\text{NO}_5\text{Si}$: C, 69.06; H, 7.53; N, 2.68. Found: C, 68.98; H, 7.62; N, 2.70.

2.2. Pseudopeptide PK-Etz synthesis

The peptidomimetic analog, Ac-Tyr-Phe-Ser- $\Psi[(E)\text{-CH=C}]$ Pro-Arg-Leu-NH $_2$ (**PK-Etz**) was synthesized manually by the solid-phase method, using the Fmoc-strategy and starting from 0.1 mM Rink amide resin (Novabiochem, 0.47 mM/g). The Fmoc protecting group was removed by 20% piperidine in DMF and the resin and later on the growing peptide-resin was washed with DMF, the MeOH and DCM. A fivefold excess of the respective Fmoc-amino acids was activated *in situ* using HBTU (0.9 equiv.)/HOBt (1 equiv.) in NMP and coupling reactions were base catalyzed with collidine. Amino acid side-chain protecting groups were TBDMS for Tyr and Ser(OTBDMS) $\Psi[(E)\text{-CH=C}]$ Pro and Pbf for Arg. The synthesis of enantiomerically pure Fmoc-Ser(OTBDMS) $\Psi[(E)\text{-CH=C}]$ Pro-OH is described above. The coupling of this Ser-*trans*-Pro isostere was

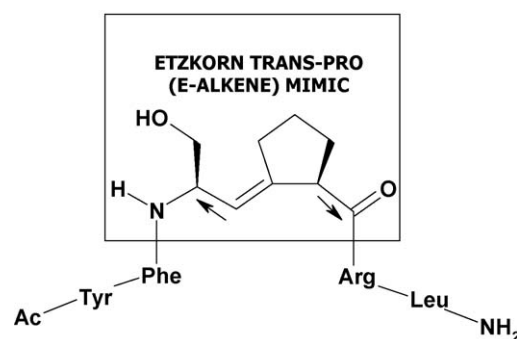


Fig. 1. Structure of the analog **PK-Etz** (Ac-Tyr-Phe-Ser- $\Psi[(E)\text{-CH=C}]$ Pro-Arg-Leu-NH $_2$), containing an (*E*)-alkene, *trans*-Pro isostere ('Etzkorn'). In this motif, the peptide bond that binds the amino group of the Pro is locked into a *trans* orientation by replacement with a double bond, which lacks the ability to rotate between *trans* and *cis* orientations as does a normal peptide bond [33,34].

mediated by HATU/HOAt instead of HBTU/HOBt. The completeness of each coupling reaction during synthesis was monitored by the Kaiser test. A second coupling was performed when the test was found positive. Cleavage of the peptide from the resin with side-chain deprotection was performed by treatment with TFA:H $_2$ O:TIS (95.5:2.5:2.5, v/v/v, 10 ml/g peptide-resin) for 1.5 h. The cleaved peptide was precipitated with 20 volumes of diethyl ether, filtered, washed successively with more ether and air-dried. The resulting crude peptide was extracted with water and lyophilized (Fig. 1).

The peptidomimetic analog was purified on a Waters C $_{18}$ Sep Pak cartridge, and a Delta-Pak C $_{18}$ reverse-phase column (8 mm \times 100 mm, 15 μm particle size, 100 Å pore size) on a Waters 510 HPLC controlled with a Millennium 2010 chromatography manager system (Waters, Milford, MA) with detection at 214 nm at ambient temperature. Solvent A = 0.1% aqueous trifluoroacetic acid (TFA); Solvent B = 80% aqueous acetonitrile containing 0.1% TFA. Conditions: initial solvent consisting of 20% B was followed by the Waters linear program to 100% B over 40 min; flow rate, 2 ml/min. Delta-Pak C $_{18}$ retention time: t_R = 11.3 min. Amino acid analysis was carried out under previously reported conditions [24] and used to quantify the peptides and to confirm identity, leading to the following analysis: F[1.0], L[0.9], R[0.8], Y[1.0]. The identities of the peptide analogs were confirmed via MALDI-MS on a Kratos Kompact Probe MALDI-MS machine (Kratos Analytical, Ltd., Manchester, UK) with the presence of the molecular ion 806.4 [MH $^+$].

2.3. Pheromonotropic bioassay

The pheromonotropic bioassay was performed with *H. peltigera* as described previously [3]. Stimulatory (e.g., agonistic) activity of the **PK-Etz** and the LPK derived parent peptide 1559 was determined by monitoring their ability to induce sex pheromone biosynthesis at 1, 10, 100 pmol and 1 nmol. Females injected with 1 pmol PBAN served as a reference for stimulatory activity. The pheromone content in buffer-injected moths did not exceed 10 ng/female. The pheromone glands were excised 2 h post-injection and sex pheromone was extracted and quantified by capillary gas chromatography as described previously [3]. All experiments were performed with 9–10 females per treatment.

2.4. Melanotropic bioassay

The melanotropic bioassay was performed as described previously [6]. Melanotropic stimulatory activity of the **PK-Etz** or the LPK derived parent peptide 1559 was determined by evaluating their ability to induce cuticular melanization in larvae at 1, 10, 100 pmol and 1 nmol. Larvae injected with 5 pmol PBAN

served as a reference for stimulatory activity and those injected with 50 mM HEPES, pH 7.6 served to determine the basal cuticular melanization of the ligated insects. Each experiment also involved analysis of the intensity of the melanized area in untreated and ligated larvae. The cuticular melanization was quantified as the ratio between the optical density and the scanned cuticular area (in millimeters) and was compared between control and experimental animals. All experiments were performed with 8–10 larvae per treatment.

2.5. Pupariation bioassay

The test was performed as described by Žd'árek [36]. Briefly, the tested material was injected at doses of 0.5, 5, 50 and 500 pmol into fleshfly larvae (*N. bullata*) at the early-RS stage that previously had been immobilized by chilling on ice. Control larvae were injected with water only. After removal from the ice the injected larvae were kept at 25 °C in Petri dishes lined with dry filter paper, and the time of retraction (R), contraction (C) and tanning (T) was recorded. At the end of the RS stage the larva stops crawling and irreversibly retracts the first three front segments with the cephalopharyngeal apparatus ('the mouth hooks') (retraction—R); it then contracts longitudinally to become the barrel-shaped puparium (contraction—C) and its surface becomes smooth by shrinking of the cuticle, until it attains the shape of the 'white puparium' (WP). Some 50–60 min after C the WP starts to change color by phenolic tanning of the cuticle (T) and turns to an 'orange puparium'. The effects of LPK, the LPK C-terminal pentapeptide and/or **PK-Etz** were expressed as a difference between the control and experimental larvae, in the mean time between the occurrences of C and T. Eight to 12 larvae in each group were injected, and the test was repeated four times. Larvae were injected by means of a disposable calibrated glass capillary with a pointed tip. The volumes of injected solution ranged from 0.5 to 1.0 µl. The definition of a threshold dose was the dose that demonstrated differences of at least 25% from the control group in R, C and T in each of the four trials.

2.6. Myotropic bioassay

Hindguts of adult *L. maderae* cockroaches were separated from the central nervous system (CNS) and dissected [12], suspended in a 5 ml chamber, and prepared for recording as previously described [9]. Threshold concentrations are determined by adding a known quantity of analog (dissolved in 0.5 ml of bioassay saline) to the bioassay chamber containing the hindgut. The threshold concentration is defined as the minimum concentration of analog required to elicit an observable change in the frequency (50%) or amplitude (10%) of contractions within 1 min and sustained for 3 min. Threshold concentrations would be obtained from measurements of three to five cockroach hindguts on consecutive days.

2.7. Statistical analysis

The results of the pheromonotropic and melanotropic assays were subjected to one-way ANOVA. All data are presented as mean ± standard error mean. The significance of differences among means was evaluated with the Tukey–Kramer HSD (honestly significant difference) test at $P < 0.05$. All statistical analyses were calculated using JMP version 5.1.2, 2004®, SAS Institute Inc., Cary, NC, USA.

3. Results

3.1. Pheromonotropic bioassay

The results of a dose response evaluation of the PK/PBAN analog **PK-Etz**, containing the (E)-alkene *trans*-Pro isosteric component, a

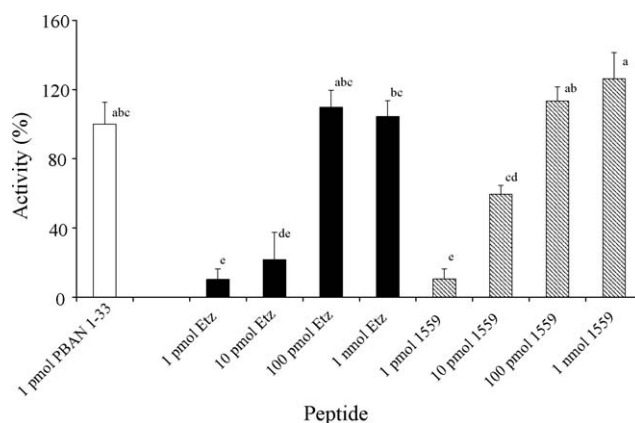


Fig. 2. *In vivo* dose–response agonist pheromonotropic activity of **PK-Etz**, the parent peptide 1559 and PBAN in *H. peltigera* females. Activity is expressed as the ratio (as a percentage) between the pheromone content elicited by the injection of each of the peptides at the listed doses and by PBAN at 1 pmol (defined as 100%) ± SEM of 9–10 samples. Statistical analysis compared differences between the pheromonotropic agonistic activities of the tested peptides. Different letters indicate significant differences in activity at $P < 0.05$.

parent peptide 1559, and PBAN1-33 as agonists in the *in vivo* pheromonotropic assay in *H. peltigera* are illustrated in Fig. 2. While less potent than the full-length PBAN 1-33, **PK-Etz** nonetheless matches the potency of the parent peptide 1559 at 1, 100 and 1000 pmol, and the difference at 10 pmol is not statistically significant. Both **PK-Etz** and 1559 demonstrate an equal response at 100 pmol and the data suggests that the threshold response is between 10 and 100 pmol. It should be noted that C-terminal pentapeptide core fragment-analogs in which the variable X position is occupied by either an S or a T show similar pheromonotropic activity in the heliothine moths *Helicoverpa zea* [1].

3.2. Melanotropic bioassay

In the melanotropic bioassay in *S. littoralis*, the analog **PK-Etz** is an extremely potent agonist capable of stimulating melanin formation at all tested concentrations (Fig. 3). Indeed, **PK-Etz** matches the potency of the natural elicitor PBAN1-33, and exceeds the maximal response (efficacy) by a factor that ranges between

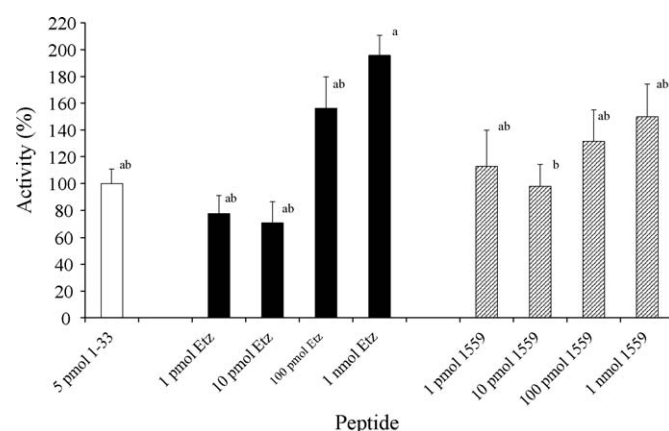


Fig. 3. *In vivo* dose–response agonist melanotropic activity of **PK-Etz**, the parent peptide 1559 and PBAN in *S. littoralis* larvae. Activity is expressed as the ratio (as a percentage) between the extents of melanization elicited by the injection of each of the peptides at the listed doses and by PBAN at 5 pmol (defined as 100%) ± SEM of 8–10 samples. Statistical analysis compared differences between the melanotropic agonistic activities of the tested peptides. Different letters indicate significant differences in activity at $P < 0.05$.

Table 1

Sequences of the compounds evaluated in the four PK/PBAN bioassays.

Name	Sequence
PBAN1-33	LADDMPATPADQEMYRQDPEQIDSRRTKYFSPRLa
1559	Ac-YFTPRLa
LPK	pQTSFTPRLa
[4–8] LPK	FTPRLa
PK-Etz	Ac-YFS-Ψ[trans-CH=C]PRLa

Ac: acetyl; Etz: *trans*-Pro Etzkorn mimetic component (see Fig. 1).**Table 2**Pupariation acceleration (*Neobellieria bullata*) and hindgut contractile (*Leucophaea maderae*) activity of PK/PBAN analogs LPK [4–8] LPK and *trans*-Pro mimetic **PK-Etz**.

PK/PBAN Analogs		Threshold	
		Pupariation [pmoles]	Hindgut contraction [10^{-8} M] ^a
LPK	pQTSFTPRLa	0.3 [25]	0.1 ± 0.05 [20]
[4–8] LPK	FTPRLa	0.3 [25]	0.2 ± 0.03 [20]
PK-Etz	Ac-YFS-Ψ[trans-CH=C]PRLa	0.3	0.2 ± 0.04

^a Threshold concentrations for hindgut contractile activity in the table are equivalent to 5, 10 and 10 pmol for LPK [4–8] LPK and PK-Etz, respectively.

1.5 and 1.9 at doses of 100 pmol and 1 nmol. **PK-Etz** also matches the potency of the fragment-analog 1559. The efficacy of 1559 is not statistically different from that of PBAN1-33 (Table 1).

3.3. Pupariation and myotropic bioassays

The results of an evaluation of *trans*-Pro isosteric analog **PK-Etz** in the larval pupariation assay of *N. bullata* and isolated hindgut contraction assay of the cockroach *L. maderae* are summarized in Table 2. The PK LPK (Lem-PK) is native to the *L. maderae* cockroach and also the first PK sequence used to establish the pupariation acceleration effect in larvae of the flesh fly *N. bullata* and is used as the control peptide. Analog **PK-Etz** is equipotent with both LPK and its C-terminal pentapeptide fragment-analog [4–8] LPK in the pupariation bioassay, demonstrating a potent threshold dose of 0.3 pmol. In the *L. maderae* hindgut contractile bioassay, **PK-Etz** is equipotent with the LPK C-terminal pentapeptide ([4–8] LPK), with a threshold concentration of 2×10^{-9} M, and is statistically equivalent to the potency of the natural LPK (1×10^{-9} M) (Table 2).

4. Discussion

The C-terminal pentapeptide FXPRLa is highly conserved and thus, shared by PBAN and other pyrokinins. This pentapeptide has further been identified as the active core in pheromone bioassays ($X = S$) [2,16,25,27] and in an expressed PBAN receptor assay from the moth *Heliothis virescens* [14] and *S. littoralis* [37], although the C-terminal hexapeptide YFXPRLa ($X = S$) exhibits much greater potency [3,11]. In the pheromone bioassay of the heliothine insect *H. zea* the core PK/PBAN C-terminal pentapeptide sequence exhibits similar potency whether the variable X position is occupied by an S or a T [1]. The C-terminal pentapeptide common to the PK/PBAN class has also been found to retain significant activity in other bioassays, such as melanotropic, pupariation and hindgut myotropic preparations.

Several turn conformations have been proposed for the core pentapeptide region based on NMR experiments of the pyrokinin PBAN and/or core analogs in solution. Using the C-terminal hexapeptide PBAN analog [D-Phe²⁹ [28–33]] PBAN in an NMR solution conformation study, Wang et al. reported that it adopts a type II β-turn. However, the authors concluded that this

observation may result from conformational averaging of a type I β-turn and an extended structure [32]. Clark and Prestwich investigated the solution conformation of the natural HezPBAN and reported a type I' β-turn with a *cis*-Pro in the C-terminal pentapeptide region [8]. Among drawbacks to the studies conducted by Wang et al. and Clark and Prestwich are that they were investigating highly flexible structures and NMR experiments were conducted in solutions incorporating organic solvents, which can promote formation of secondary structure that is not necessarily relevant to the conformation adopted at the receptor site. Nachman et al. conducted a conformational study of the rigid, cyclic PK/PBAN analog *cyclo*[NTSFTPRL] (*cyclo*[Asn¹]LPK) in aqueous solution containing no organic solvents using a combination of NMR spectroscopic and molecular dynamics calculations [21,23]. The specific conformation of this constrained, cyclic analog in aqueous solution was shown to be extremely rigid, featuring a *trans*-oriented Pro in the second position of a type-I β-turn over residues Thr-Pro-Arg-Leu within the core region. A *trans*-Pro is a defining characteristic of a type I β-turn [7]. The very large (for Thr-2, Thr-5, and Leu-8) and very small (for Ser-3 and Arg-7) coupling constants found indicated that the backbone of *cyclo*[Asn¹]LPK was rigidly held in a single or a few closely related conformations, since conformational averaging would have given averaged, intermediate values [23]. Recently, a structure for the HezPBAN receptor has been predicted using the X-ray diffraction structure of the GPCR rhodopsin as a template; and this calculated structure has been used to build a binding model for the HezPBAN C-terminal hexapeptide fragment adopting each of the three proposed β-turn types. The model clearly supports the presence of a β-turn in the receptor bound conformation of PBAN core, but is not precise enough to provide evidence for the specific type of β-turn [31].

Despite the conformational constraint imposed upon the cyclic PK/PBAN analog *cyclo*[Asn¹]LPK, it was found to retain 10% of the pheromone activity of the 33-residue Bom-PBAN-I in a pheromone bioassay in the silkworm *B. mori* [21], the same percentage of activity retained by the linear C-terminal PBAN hexapeptide. The analog *cyclo*[Asn¹]LPK was also found to retain significant bioactivity in several other PK/PBAN bioassays, including hindgut contractile (cockroach *L. maderae*) [23], inducible contractile (cockroach *L. maderae*) [22], egg diapause induction (silk worm *B. mori*) [22], and pupariation (flesh fly *N. bullata*) [36] assay systems.

In order to provide more definitive evidence that a *trans*-Pro, and a type I β-turn, represented the active conformation for the PK/PBAN neuropeptide class, the PK/PBAN analog **PK-Etz**, incorporating a *trans*-Pro isostere, was evaluated in four diverse PK/PBAN bioassay systems. These bioassays were the pheromone biosynthesis assay in the moth *H. peltigera*, the melanization assay in the Egyptian cotton leaf worm *S. littoralis*, the pupariation assay in the fleshfly, *N. bullata*, and the hindgut myotropic assay in the cockroach *L. maderae*. In **PK-Etz**, the peptide bond of the Pro is replaced with a rigid double bond that locks in the *trans* orientation [33] (Fig. 1). Analog **PK-Etz** demonstrated activity essentially equivalent to parent PK/PBAN analogs of equal length in all four bioassay systems. In the melanization, pupariation and hindgut contractile assays, **PK-Etz** matched or approached the activity of natural PK/PBANs isomorphs. Of particular note is the fact that **PK-Etz** exceeded the efficacy (maximal response) of the natural PBAN1-33 in the melanotropic bioassay by close to a factor of 2 (at 1 nmol).

The relatively potent agonist activity of **PK-Etz** provides strong evidence that a *trans*-Pro represents an important conformational aspect of the interaction of PBAN with its receptor in the four disparate PK/PBAN bioassay systems, each representing a different insect species. This conclusion, at least

in the case of the melanotropic process, is further supported by the activity profile of a PK/PBAN analog that incorporates a novel dihydroimidazoline moiety, recently proposed as a *trans*-Pro isostere [17].

Establishment of a *trans*-Pro orientation provides valuable evidence for the identity of the active PK/PBAN conformation. Three previous studies have led to the proposal of different β -turn types (type I, type II and type I') for the PK/PBAN core region (as discussed above). Of these studies, only Nachman et al. [21,23] used both a conformationally rigid PK/PBAN analog along with aqueous solutions free of added organic solvents that artificially promote the formation of secondary structure. Of note is the fact that Wang et al. [32] admit that their observation of a type II β -turn could have been the result of conformational averaging of a type I β -turn (identified in the study by Nachman et al.) and an extended conformation in the flexible analog used. The type I β -turn proposed by Nachman et al. features a *trans*-Pro that was clearly evident in the rigid, cyclic analog *cyclo*[Asn¹]LPK and has now been confirmed by the potent activity of **PK-Etz**, which locks in a *trans* orientation with an alkene bond that is unable to rotate. This finding is not consistent with a type I' β -turn proposed in the study by Clark and Prestwich [8] that used the highly flexible HezPBAN, as this turn type features a *cis*-Pro rather than a *trans*-Pro. The work described here not only provides evidence for the orientation of Pro and core conformation for the interaction of PK/PBAN neuropeptides with receptors associated with a broad range of PK/PBAN-regulated processes, but also identifies a scaffold with which to design mimetic analogs of this peptide class. Such analogs may provide leads in the development of novel insect-specific, environmentally favorable pest management agents capable of disrupting PK/PBAN-regulated physiological systems.

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